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Rapid separation of solasodine glycosides by an immunoaffinity column using anti-solamargine monoclonal antibody

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Abstract

Immunoaffinity column using anti-solamargine monoclonal antibody for separation of solasodine glycosides was established. This method was specific for solasodine glycosides which was detected by thin layer chromatography and the western blotting. Total solasodine glycosides have been separated directly from the crude extract of *Solanum khasianum* fruit by the newly established immunoaffinity column.

Abbreviations: MAb, monoclonal antibody; ELISA, enzyme-linked immunosorbent assay; MALDI, matrix-assisted laser desorption/ionization; HSA, human serum albumin; PBS, phosphate buffer saline; TPBS, PBS containing 0.05% of Tween 20; S-PBS, PBS containing 5% skim milk; ABTS, 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt; TLC, thin layer chromatography; PVDF, polyvinylidene difluoride

Introduction

The most important feature of solasodine is that it can be converted to dehydropregnenolone. Solasodine is found with a series of sugar residues attached to the oxygen at the C-3 position. By far the most common forms are the triglycosides, solamargine being predominant (Mahoto et al., 1980). Therefore, the steroidal alkaloid glycosides of solasodine type like solamargine have become important as a starting material for the production of steroidal hormones in the pharmaceutical and medical areas. In previous paper, we presented the method of direct determination for antigen conjugate by matrix-assisted laser desorption/ionization (MALDI) mass spectrometry (Shoyama et al., 1993a, b; Goto et al., 1994). We already set up the production of monoclonal antibody (MAb) against solamargine and the practical application of enzyme-linked immunosorbent assay (ELISA) for determination solarmargine contained in *Solanum* spp. plants (Ishiyama et al., 1996), in order to select the strain of higher yielding steroidal alkaloid glycosides because rapid, simple, highly sensitive and reproducible assay systems are required for a large number of plants, and a limited small amount of samples. We further reported a simple method for determination solasodine glycosides by the western blotting using MAb against solamargine (Tanaka et al., 1997). As an extension of this approach, we present here the production of the immunoaffinity column using anti-solamargine MAb for one-step-separation of total steroidal alkaloid glycosides.

Materials and methods

Chemicals, immunochemicals and plant materials

HSA was provided by Pierce (Rockford, IL, U.S.A.). Peroxidase labeled anti-mouse IgG was provided by Organon Teknika Cappel Products (West Chester, PA, U.S.A.). PVDF membrane (Immobilon-N) was purchased from Whatman International Ltd. (Maidstone, U.K.). All other chemicals were standard commercial products of analytical grade.

Fruit of *Solanum khasianum* was obtained from Faculty of Pharmaceutical Sciences, Nagasaki University, Japan. Solamargine and solasonine were isolated from fresh fruits of *S. khasianum* as previously described (Mahato et al., 1980).

An immunogen, solamargine-HSA conjugate, was synthesized from solamargine by $NaIO_4$ treatment followed by conjugation with HSA as reported previously (Weiler et al., 1976) with modifications. Immunization and hybridization were carried out as reported (Ishiyama et al., 1996) in this laboratory.

Purification of anti-solamargine MAb

One of the original clones (SMG-BD9) producing anti-solamargine MAb (Ishiyama et al., 1996) was purified by using a Protein G Sepharose 4FF column (0.46×11 cm, Amersham Pharmacia Biotech, Uppsala, Sweden) as previously reported with modification (Langone, 1982). The absorbed MAb was eluted with 0.1 M glycine buffer (pH 3.0), the eluate was neutralized with 1 M tris buffer solution, and dialyzed 5 times against H_2O and finally lyophilized to give MAb.

Confirmation of purity of MAb by MALDI mass spectrometry

A small amount of the purified MAb was mixed with a 10^3-fold molar excess of sinapic acid in an aqueous solution containing 10% (w/v) trifluoroacetic acid. The mixture was inserted into a JMS-LDI 1700 time-of-flight mass monitor (JEOL, Japan) and irradiated with a N_2-laser (337 nm, 3 ns pulse). The ions formed by each pulse were accelerated by a 30 kv potential in a 1.7 m evacuated tube. The data were analyzed using a compatible computer.

Preparation of immunoaffinity column using anti-solamargine MAb

Purified MAb (2 mg) was dialyzed against coupling buffer, pH 5.5, overnight at 4 °C and after that MAb was oxidized with $NaIO_4$. Performed the MAb oxidation in container covered with aluminium foil and then mixed gently for 1 h at room temperature. Immediately after oxidation, added glycerol at a final concentration of 20 mM and mixed for 10 min and then dialyzed against coupling buffer, pH 5.5 at 4 °C. Oxidized MAb was added to a slurry of Affi-Gel Hz hydrazide gel (2 ml gel volume, Bio-Rad) in coupling buffer and coupled by stirring at room temperature for 24 h. The eluant and washing solution (20 mM phosphate buffer, 0.5 M NaCl, pH 7.0) were combined, and the unbound protein was measured at 280 nm for determining coupling efficiency, which was found to be 55.4% of the MAb added. The immunoaffinity gel was washed with PBS and packed in plastic mini-column. The gel was equilibrated with PBS which contained 0.02% sodium azide and stored at 4 °C.

Protocol for immunoaffinity column chromatography

The procedure was carried out at room temperature, except incubation. The immunoaffinity column was washed with PBS before use. A sample dissolved in PBS was loaded onto the immunoaffinity column. The loaded column was incubated at 4 °C for 2 h with the flow stopped, and then washed with PBS (25 ml). The column was eluted with 40% methanol in PBS (15 ml). After elution of solasodine glycosides, the immunoaffinity column was washed with PBS, equilibrated with PBS containing 0.02% of sodium azide, and then stored at 4 °C until subsequent use.

Competitive ELISA

The individual fractions were determined by ELISA as reported previously (Ishiyama et al., 1996) with modification. A 96 well-immunoplate to which had been adsorbed by 50 μl of 2 μg ml^{-1} solamargine-HSA was treated with 300 μl PBS containing 5% skim milk (S-PBS). The plate was washed 3 times with PBS containing 0.05% tween 20 (TPBS). Fifty μl samples diluted with 20% methanol were added to the above well. Fifty μl of MAb (100 ng ml^{-1}) diluted with T-PBS was further added to the well and then incubated for 1 h. The plate was mixed for 1 min. The plate was washed 3 times with TPBS. The plate was incubated with 100 μl of 1000 times-diluted peroxidase anti-mouse IgG for 1 h. After washing the plate 3 times with TPBS, 100 μl of substrate solution, 0.1 M citrate buffer (pH 4.0) containing 0.006% hydrogen peroxide and 0.3 mg/ml ABTS, were added to each well and incubated for 15 min. Absorbance at 405 nm was measured with a FAR 400 Electrophotometer (SLT-LABINTRUMENTS, Salzburg, Austria). All the reaction were carried out at 37 °C.

Western blotting

The fractions were applied to a TLC plate and developed with $CHCl_3$/MeOH/NH_3 (6:3:1). The developed TLC plate was dried and then sprayed with a

blotting solution mixture of isopropanol/methanol/water (1:4:8, by vol.). It was placed on a stainless-steel plate, and then covered with a PVDF membrane sheet. After covering with a glass micro fiber filter sheet, the whole was press evenly 45 s with a 120 °C hot plate as previously described (Tanaka et al., 1997; Taki et al., 1994) with modification. The PVDF membrane was separated from the plate and dried.

The blotted PVDF was dipped in water containing $NaIO_4$ (10 mg ml^{-1}) at room temperature for 1 h. After washing with water, 50 mM carbonate buffer solution (pH 9.6) containing BSA (1%) was added, and stirred at room temperature for 3 h. The PVDF membrane was washed twice with TPBS for 5 min, and then washed with water. The PVDF was immersed in anti-solamargine MAb, stirred at room temperature for 1 h. After washing the PVDF membrane twice with TPBS and then water, 1000 times dilution of peroxidase-labeled goat anti-mouse IgG in G-PBS was added and stirred for 1 h. The PVDF membrane was washed twice with TPBS and water, then exposed to 1 mg ml^{-1} 4-chloro-1-naphol 0.03% hydrogen peroxide in PBS solution which was freshly prepared before use for 10 min at room temperature, and the reaction was stopped by washing with water. The immunostained PVDF membrane was allowed to dry.

Determination of absorption and elution for immunoaffinity column

The capacity of immunoaffinity column were determined. The immunoaffinity column (2 ml of gel) was loaded with solamargine (40 μg), solasonine (40 μg) and solasodine (20 μg) in PBS which was added separately. The column was incubated at 4 °C for 2 h, washed with PBS, and then monitored by ELISA until the substance disappeared. The immunoaffinity column loaded was eluted with 40% methanol in PBS, and the concentration of each fraction (1 ml) was analyzed by ELISA.

Purification of steroidal alkaloid glycosides by immunoaffinity column

The dried powder of *S. khasianum* fruit (20 mg) was extracted with methanol (0.5 ml) 5 times using ultrasonic bath 15 min. After filtered with 0.45 μm filter, filtrate was dried under N_2 stream and vacuum drying oven. The residue was redissolved with methanol and diluted with PBS. The solution was loaded on the immunoaffinity column and stood at 4 °C for 2 h. The column was washed with the washing buffer solution. After solasodine glycosides had disappeared, the column was eluted with 40% methanol in PBS at 0.1 ml min^{-1}. The total solasodine glycosides concentration was assayed by ELISA. Solasodine glycosides were developed by TLC, and then sprayed with H_2SO_4 compared with the western blotting on PVDF membrane.

Result

Purify MAb

After MAb from the original clone (SMG-BD9) was purified by Protein G Sepharose 4FF column, the MALDI mass spectrometry was measured to confirm the purity of the MAb (SMG-BD9) as previously reported (Sakata et al., 1994). The molecular weight was 148 700, which is in good agreement with that of human IgG1 being determined as 146 000 (Langone, 1982). It became evident that the purified MAb using protein G column can be used for the affinity column.

Immunoaffinity column chromatography

The purified MAb was coupled to Affi-Gel Hz hydrazide gel to give an immunoaffinity gel (7.54 μmol of MAb/2 ml gel). The elution system for the immunoaffinity column was investigated by using various elution buffers (data not shown). It appeared that PBS containing 40% methanol was most effective and suitable for elution of solasodine glycosides.

To assess the capacities and the recoveries of solamargine, solasonine and solasodine from the immunoaffinity column, each substance was added separately and run through the column. The content of individual fractions were determined by ELISA after washing with PBS, and then completely eluting with PBS containing 40% methanol. The established elution buffer system resulted in 95.31, 97.20 and 95.80% recovery of solamargine, solasonine and solasodine, respectively. The capacity of immunoaffinity column was determined to be 6.19, 12.92, 3.92 μg of solamargine, solasonine and solasodine, respectively per ml of immunoaffinity gel.

Separation of steroidal alkaloid glycosides from crude extract of S. khasianum fruit by the immunoaffinity column

The crude extract of *S. khasianum* fruit was loaded on the immunoaffinity column, washed with PBS

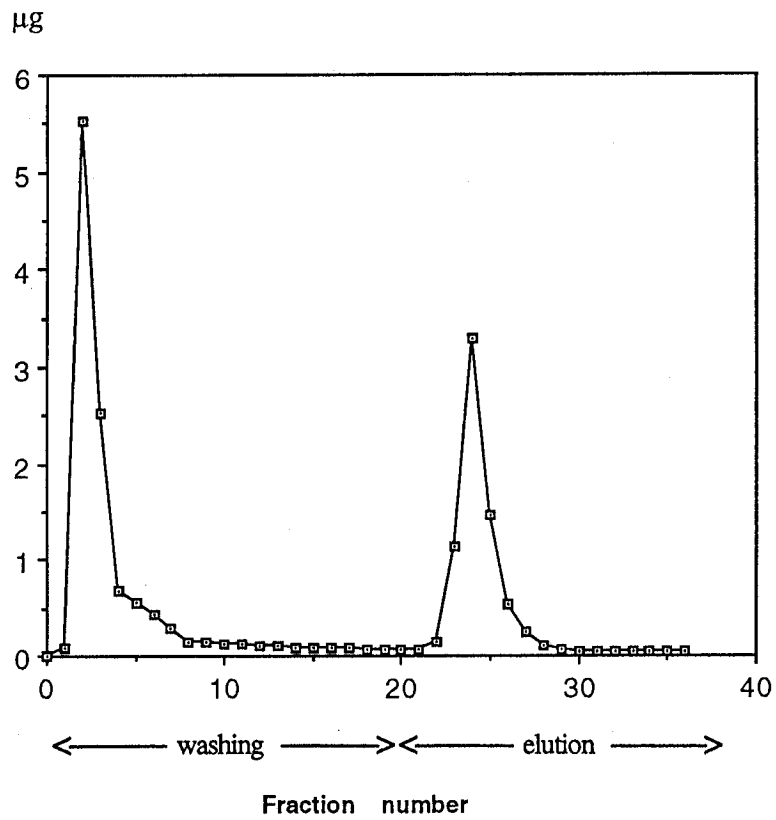

Figure 1. Elution profile of solasodine glycosides using the affinity column from the crude extract of *S. khasianum* fruit. The column loaded was washed with PBS, then eluted with PBS containing 40% MeOH.

and eluted with 40% methanol in PBS. Figure 1 shows a chromatogram detected by ELISA. Fraction 1–8 contained over loaded solasodine glycosides like solamargine, solasonine, L-rhamnosyl-(1→4)-O-3-β-D-glucopyranosyl solasodine and the other non-related unknown compounds which were detected by TLC stained with H_2SO_4 as indicated in Figure 2b. The peak of fractions 22–29 shows the elution of total solasodine glycosides eluted with 40% methanol in PBS. The fractions contained only solamargine, solasonine and L-rhamnosyl-(1→4)-O-3-β-D-glucopyranosyl solasodine which were determined by TLC stained with H_2SO_4 and the western blotting (Figure 2a, b). The above over loaded solasodine glycosides can be separated completely from the non-related unknown compounds by repeated column chromatography (data not shown). This result indicates that solasodine-type steroidal alkaloids can be separated by the newly established immunoaffinity column.

Discussion

The separation method for solasodine glycosides using an immunoaffinity column was established. Previously we reported PBS solution containing 45% methanol cleaved the antigen-antibody complex in the case of forskolin, and the antibody in immunoaffinity column was stable even in the elution solution (Yanagihara et al., 1996). In this investigation, PBS solution containing 40% methanol was used for elution buffer which shows most suitable for separation. The stability of antibody against PBS containing 40% methanol is high since the immunoaffinity column can be used over 10 times or more under the same condition without any substantial loss of capacity. The capacity of column for solasodine was determined to be 3.92 μg resulting in lower compared to solamargine (6.19 μg) and solasonine (12.92 μg) due to smaller cross-reactivity (44%) with anti-solamargine MAb (Ishiyama et al., 1996). Since all solasodine glycosides can be cross-reacted with anti-solamargine MAb (Ishiyama et al., 1996; Tanaka et al., 1997), it is

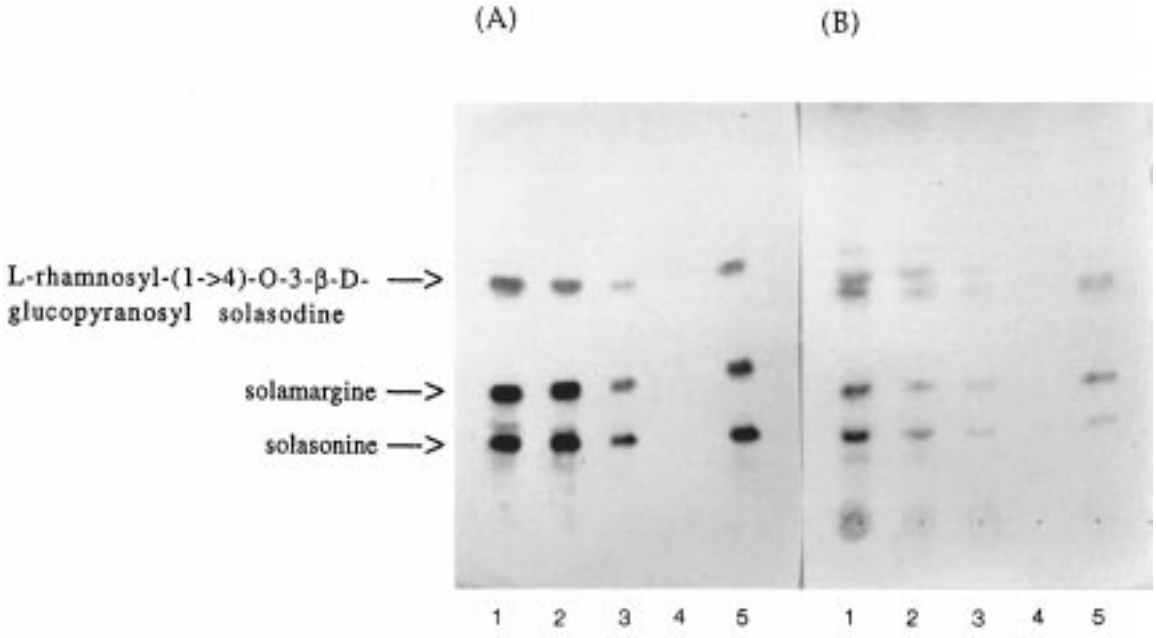

Figure 2. The profiles of western blotting (A) and TLC stained with H_2SO_4(B) separated by the affinity column as indicated in Figure 1. (1) Crude extract of *S. khasianum* fruit, (2–4) washing fractions with PBS, (5) eluting fraction with PBS containing 40% MeOH.

pointed out that the mixture of total solasodine glycosides can be separated by the immunoaffinity column. This wide-cross reaction is the major advantage of this MAb reagent used in this immunoaffinity column. Therefore, this method can be available for the rapid and simple separation of total solasodine glycosides.

To expand this method, the crude extract of *S. khasianum* fruit was subjected onto the immunoaffinity column. In order to confirm the evidence of solasodine glycosides elution, TLC stained with H_2SO_4 and the western blotting were carried out to indicate the clear separation profile. It becomes clear that a single immunoaffinity column can separate only solasodine glycosides which have been important for the starting materials of steroidal hormones from the crude extract of *S. khasianum* fruit.

This is the first report in which an immunoaffinity column for the separation of solasodine glycosides and its application has been described. This system can concentrate and separate the total solasodine glycosides from plant materials. Therefore, this methodology can be available for the detection of higher yielding solasodine glycosides plantlets *in vitro* of *S. khasianum* by the combination with ELISA because the regenerated plantlets contain a small amount of solasodine glycosides.

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